Fermentation of Long Chain Compounds by *Torulopsis magnoliae*. III. Preparation of Dicarboxylic Acids from Hydroxy Fatty Acid Sophorosides[1]

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Abstract

Conversion of hydroxy fatty acids, prepared from the products of fermentation of long chain hydrocarbons or fatty acids with *Torulopsis magnoliae*, to dicarboxylic acids with 15 to 18 carbon atoms is described. Both nitric acid oxidation and fission in 85% KOH give yields of 60–75%, but the products have different compositions. The nature of the compound fermented determines the composition of the hydroxy acids produced and hence that of the derived dicarboxylic acids. A convenient method is presented for hydrolysis of the hydroxy fatty acid sophorosides obtained by fermentation.

Introduction

As part of a study of methods of modifying long chain compounds the yeast *Torulopsis magnoliae* was used to convert them to hydroxy fatty acid glycosides of the disaccharide sophorose (7,9). Hydroxy fatty acids which have a hydroxyl group at the penultimate (ω-1) or terminal (ω) positions are obtained by acid hydrolysis of the glycosides. It was considered that the fermentation process would be more valuable if a study were made of some reactions of the hydroxy acids which involved conversion to other difunctional compounds. The only hydroxy acid which has been used to any extent in chemical reactions is 12-hydroxystearic acid, obtained from hydrogenated castor oil. However, a hydroxy acid with the hydroxyl group almost at the end of the chain might be expected to react in a different way from one with the group near the center of the chain.

An investigation has now been made of methods of converting these hydroxy acids into long chain dicarboxylic acids which are not otherwise readily available. In the present study the hydroxy fatty acid portions of two typical fermentation products have been used; firstly, a mixture consisting mainly of 16-hydroxyheptadecanoic and 17-hydroxyoctadecanoic acids, obtained by fermenting a straight chain hydrocarbon fraction ("distillate wax"), and secondly, crude 17-hydroxyoleic acid obtained by fermenting a commercial oleic acid. Methods reported by Steadman and Peterson (8) for the preparation of dicarboxylic acids from 12-hydroxystearic acid, by nitric acid oxidation and fission in strong alkali, have been reexamined and modified for use with the hydroxy acids obtained by fermentation. In addition, a number of hydrolysis procedures have been tried to obtain maximum yields of hydroxy fatty acids from the sophorosides.

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Experimental

Emery oleic acid (Emersol 233 LL), which when analyzed by GLC had the composition 14:0, 2; 14:1, 2; 16:0, 4; 16:1, 12; 17:0, 1; 18:0, 1; 18:1, 75; 18:2, 3, was used for fermentation. The "distillate wax" hydrocarbon cut was found, using octacosane as internal standard, to contain 80% of straight chain hydrocarbons with the composition C_{20}, 2; C_{21}, 8; C_{22}, 18; C_{23}, 22; C_{24}, 23; C_{25}, 18; C_{26}, 7; C_{27}, 1.

Gas-Liquid Chromatography

Using mixtures of pure methyl esters of 15-hydroxy- and 16-hydroxypalmitic acids, 17-hydroxystearic, 17-hydroxyoleic, and 18-hydroxystearic acids and the corresponding acetylated esters, it was found, for both silicone and polyester columns, that quantitative GLC results were only obtained when acetoxy esters were used. When unacetylated esters were tried the response of ω-hydroxy ester on the silicone column was about 60% of that of ω-1 hydroxy ester, and on the polyester column both types of hydroxy ester responded poorly. The GLC units were of conventional design using thermal conductivity cells for detection. The silicone column was a 30 in. x ¼ in. copper column packed with silicone SE-30 on 60–80 mesh acid-washed Celite (1:6 w/w) operated at 220C and a flow rate of 40 ml helium/minute. The polyester column was an 8 ft x 3/16 in. copper column packed with ethylene glycol succinate on 60–80 mesh acid-washed Chromosorb W (1:10 w/w) operated at 210C and a flow rate of 50 ml helium/minute.

The composition of the hydroxy esters, prepared by hydrolysis of the sophorosides, was obtained by analysis of acetoxy esters with the polyester column. This column resolved almost all the components including saturated and unsaturated acetoxy esters of the same chain length. The silicone column, though it does not resolve every component (9,10), was used to confirm the results obtained with the other column. Since esters of saturated ω-hydroxy acids and of unsaturated ω-1 hydroxy acids with one more carbon atom, present as minor components of esters (A) (Table I), had almost the same emergence times using the polyester column, esters (A) were analyzed before and after hydrogenation, and the percentages of the overlapping components were calculated by difference.

Since the crude hydroxy esters contained estolides and other nonvolatile material, an internal standard (6) was used to determine the percentage of the methyl esters which were volatile when analyzed with the silicone column. In the first place, acetoxy esters with methyl 6-acetoxypentadecanoate as standard were used, but it was found that satisfactory per cent volatile figures could be obtained with hydroxy esters and methyl 6-hydroxypentadecanoate

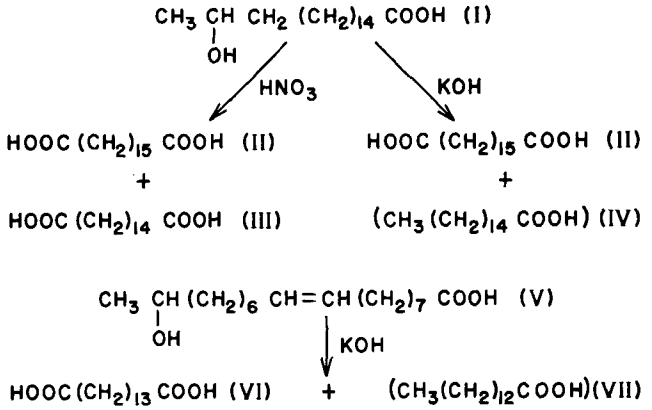


FIG. 1. Conversion of hydroxy acids to dicarboxylic acids.

(10) as standard. This simplification was possible because the mixtures under investigation contained relatively small percentages of ω-hydroxy esters.

Dicarboxylic acids were analyzed as dimethyl esters using the silicone column and also an 8 ft x 3/16 in. copper column packed with ethylene glycol phthalate (3) on 60–80 mesh acid washed firebrick (1:4.5 w/w) operated at 207C and a flow rate of 60 ml of helium/minute. Dimethyl tridecanedioate (prepared from methyl erucate) was used as internal standard in estimations of volatile material. Using the silicone column an average factor of 1.04 was applied to the C_{15}, C_{16}, C_{17} and C_{18} diesters. Using the polyester column the following factors were used: C_{13}, 1.000; C_{14}, 1.025; C_{15}, 1.050; C_{16}, 1.075; C_{17}, 1.100; C_{18}, 1.125. Tests with mixtures of pure components indicated that the analyses were accurate to within one unit percent on a weight basis.

Reaction Conditions

Fermentation. Fermentations were carried out as described previously (7). The product separated from the fermentation medium as a heavy amber-colored liquid, which consisted of sophoroside mixed with approximately its own weight of water. The product from "distillate wax" was washed with light petroleum to remove unfermentable starting material.

Hydrolysis. Hydrolysis of a batch of "distillate wax" fermentation product was carried out as follows: crude product (1300 g; equivalent to 740 g of dry glycoside) was added to 500 ml of water containing 10 ml of concentrated sulfuric acid and the mixture heated to 100C and stirred vigorously for 6 hr. At the beginning of the reaction the glycoside formed a thick syrup on the bottom of the flask but at the end the hydrolysis product appeared as a black oil on the surface of the aqueous acid. The oil was separated from the warm solution, taken up in 600 ml of methanol containing 15 ml of concentrated sulfuric acid and refluxed for 18 hr. The reaction product was poured into 2 liters of water and filtered off. The soft solid thus obtained was taken up in about one liter of ethyl acetate and washed 12 times with 15% aqueous sodium chloride. Removal of the solvent yielded 329 g of dark brown solid methyl esters. Methyl 17-hydroxy oleate, obtained from the product of the fermentation of oleic acid, was a liquid after methanolysis and could be washed free of acid without the addition of solvent.

When the aqueous acid portion obtained in the first stage of hydrolysis was neutralized with barium carbonate, barium sulfate filtered off and the filtrate evaporated to dryness, 262 g of sugar (assumed to be glucose) was obtained. Treatment of the aqueous methanol fraction from the second stage of the hydrolysis in the same way yielded 122 g of methyl glucoside (equivalent to 114 g of glucose). Since the sophoroside molecule contains two acetate groups attached to the sophorose portion (5) the product obtained by fermentation of "distillate wax" has a molecular weight of 699 (assuming a 1:1 ratio of hydroxy C_{17} and C_{18} fatty acids). Therefore, from 740 g of dry sophoroside the theoretical yield of methyl hydroxy esters is 323 g and of glucose is 382 g. No attempt was made to recover the acetic acid produced.

The crude hydroxy esters were distilled and the fraction boiling at 180–190C/0.1 mm was collected. A further quantity of volatile esters was recovered from the distillation residue by refluxing it with 5 times its weight of methanolic sulfuric acid (4%) for 18 hr and working up as before. A portion of the distilled esters was hydrogenated over Raney nickel at 150C for 4 hr.

Nitric Acid Oxidation. Hydrogenated methyl hydroxy esters (50 g) were added in 1 g portions, during 1 hr, to 350 ml of concentrated nitric acid containing 0.75 g copper and 0.30 g of ammonium metavanadate, while the mixture was stirred and maintained at 70C. The reaction was continued at 70–75C for 30 min atfer completion of the addition and then poured into 1500 ml of water. The dicarboxylic acids were filtered, washed and dried in a desiccator over NaOH. Crude acids (41 g, from oxidation of esters A) were crystallized from 350 ml of toluene (charcoal) and gave 29 g of dicarboxylic acids which were 98% volatile as methyl esters.

A portion of the crude dicarboxylic acids produced by oxidizing hydrogenated hydroxy esters, obtained from the fermentation of oleic acid, was converted to methyl esters with diazomethane. The methyl esters were separated preparatively using the silicone column and dimethyl hexadecanedioate and dimethyl heptadecanedioate were collected. The former ester had mp 51–51.5C (after crystallization from methanol) which was not depressed by an authentic sample previously prepared (9); the latter ester had mp 52–52. 5C (from methanol) which was undepressed by dimethyl heptadecanedioate prepared by the method of Blomquist et al. (1).

Alkaline Fission. Experiments following the method of Steadman and Peterson (8) were carried out at 320C in a rocking stainless steel autoclave. In the fission experiments 30 g of KOH were stirred under nitrogen in a stainless steel tube (8 in. x 1⅝ in.) which was electrically heated to 320C. Methyl hydroxy esters (10 g) were added in ½ g portions over 30 min and the reaction was continued for a further 30 min. The mixture was allowed to cool under nitrogen and taken up in 250 ml of water. The acids were liberated with concentrated hydrochloric acid, filtered and washed.

The crude acids were taken up in 30 ml of toluene (charcoal) and filtered, 20–25 ml of the toluene was distilled off and 60 ml of petroleum ether (boiling range 60–80C) added and the acids allowed to crystallize at 20C. In this way the product of the fission of esters (A) yielded 6 g acids (87% volatile as methyl esters) and that from esters (B) yielded 5.2 g acids (85% volatile as methyl esters). Pure 17-hydroxystearic acid (2 g) was also fused with 6 g of KOH in a smaller steel tube (60 ml capacity) at 320C and worked up as above. The product (2 g),

treated with diazomethane, gave methyl esters which were 94% volatile. Dimethyl heptadecanedioate was isolated by preparative GLC and had mp and mixed mp 52–53C. Acids obtained by fission of distilled hydroxy esters, derived from the fermentation of oleic acid, were also converted to methyl esters and separated by GLC. Dimethyl pentadecanedioate which had mp 43–44C (lit., 2, gives 43C) and dimethyl hexadecanedioate, mp and mixed mp 48.5–50.5C, were the principal components.

Results and Discussion

In previous work (5,9) methyl hydroxy esters were prepared from the sophorosides by acid methanolysis. This method, however, resulted in the conversion of all the sugar to methyl-$\alpha\beta$-D-glucopyranosides and make it necessary to dry the viscous syrupy sophoroside. Attempts were made to hydrolyze the sophoroside with 0.04 N sulfuric acid at 160C in a sealed tube, but when the reaction was continued until hydrolysis was complete the fatty acid portion was extensively degraded.

Direct hydrolysis with aqueous 0.4 N sulfuric acid was investigated; approximately two-thirds of the sugar was removed in this way. It is assumed that random cleavage of the two glycosidic links in the partly soluble sophoroside occurs resulting in a mixture of approximately equal parts of the hydroxy fatty acid and its insoluble glucoside. When the reaction time was increased no further hydrolysis of the glucoside took place.

Only about 25% of the partial hydrolysis product was volatile, after treatment with diazomethane, which suggested that much of the free hydroxy fatty acid had been converted to estolide by the hot dilute acid. The incompletely hydrolyzed material was then converted to hydroxy fatty acid methyl esters by acid methanolysis and the remaining one-third of the sugar was recovered as methyl glucoside. The crude esters were about 75% volatile but distillation yielded esters which were almost 100% volatile. A further quantity of esters of 70–85% volatility could be obtained by resubjecting the distillation residue to acid methanolysis to decompose estolides produced in the first distillation. The compositions of the methyl esters and the percentages obtainable, as 100% volatile esters, are shown in Table I. The structures of the components were determined as in previous work (9).

TABLE I

Composition of Hydroxy Acid Portion of Fermentation Products

	Material fermented	
	(A) "Distillate wax"	(B) Oleic acid
Hydroxy fatty acids (%)		
15 OH 16:0	3	2
15 OH 16:1	...	1
16 OH 16:0	2	4
16 OH 16:1	4	1
16 OH 17:0	39	....
16 OH 17:1	8	....
17 OH 17:0	6	....
17 OH 18:0	22	6
17 OH 18:1	10	69
17 OH 18:2	....	3
18 OH 18:0	2	....
18 OH 18:1	2	10
18 OH 18:2	....	4
18 OH 19:0	2	....
Distillable esters (%)	85	93

Nitric Acid Oxidation

Steadman and Peterson (8) reported that nitric acid oxidation converted 12-hydroxystearic acid to a mixture of dodecanedioic and undecanedioic acids. Good yields of dicarboxylic acids were obtained when their reaction conditions were used with hydrogenated hydroxy methyl esters. As shown in Fig. 1, cleavage occurs on both sides of the substituted carbon atom to yield in the case of 17-hydroxystearic acid (I), heptadecanedioic (II) and hexadecanedioic (III) acids, with the latter acid predominating. The compositions of the products, shown in Table II, are approximately those expected from the compositions of the starting materials. The smaller yield from hydroxy esters derived from "distillate wax" is partly due to the lower percentage of distillable hydroxy esters in this product.

Alkaline Fission

Fission of hydroxy acids in alkali appeared to be a valuable alternative to nitric acid oxidation since unsaturated acids can also be used, thus avoiding hydrogenation and at the same time giving different dibasic acids.

A number of workers have shown that 12-hydroxystearic acid undergoes fission in strong alkali giving dodecanedioic acid as one of the principal products. The conditions described by Steadman and Peterson (8), in which the hydroxy acid is heated in an autoclave at 330C with 30% NaOH containing some cadmium oxide, appeared to be very convenient, particularly since air could be excluded. However, when the reaction was tried with hydroxy esters from fermented oleic acid fission did not take place. The reaction was repeated with 12-hydroxystearic acid but, though some 12-oxostearic acid was produced by de-

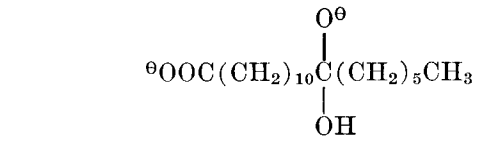
TABLE II

Products of HNO_3 Oxidation and KOH Fission

Composition of dicarboxylic acids (%)	Hydroxy esters (A) from "distillate wax"				Hydroxy esters (B) from oleic acid			
	HNO_3 oxidation	KOH fission			HNO_3 oxidation	KOH fission		
	Hydrogenated	Crude	Distilled	Hydrogenated	Hydrogenated	Crude	Distilled	Hydrogenated
C_{13}	1	1	2	....	1	1	2	1
C_{14}	5	6	9	2	4	11	8	2
C_{15}	39	12	12	4	15	54	66	5
C_{16}	40	45	43	49	53	26	18	9
C_{17}	13	32	32	39	23	8	6	66
C_{18}	2	4	2	6	4	....	....	17
Crude yield from 10 g	8.2	8.2	9.0	8.6	9.0	8.7	8.5	9.5
% Volatile as dibasic acid methyl esters	75	74	76	75	79	64	65	74
% Yield of 100% dibasic acids from crude hydroxy acids[a]	58	65	63	58	74	60	59	69

[a] In HNO_3 oxidation theoretical yields were calculated by assuming that, for esters (A) a 1 : 1 mixture of 16-hydroxy C_{17} and 17-hydroxy C_{18} methyl esters is converted to a 1:1 mixture of 15 and 16 carbon dibasic acids; and for esters (B) a 17 hydroxy C_{18} ester is converted to a 16 carbon dibasic acid. In KOH fission they were calculated by assuming that only long chain dibasic acids are produced. The % yield for the distilled and hydrogenated esters has been adjusted by taking into account the % distillable of the crude esters given in Table I.

hydrogenation, fission did not occur. The same results were obtained with 60% alkali. Fission did occur when 30% NaOH and an equimolar amount of cadmium oxide were used but the major dibasic acid produced was sebacic acid. When 85% KOH was used alone in the autoclave, reaction occurred giving a mixture of dodecanedioic, sebacic, and undecanoic acids. These products were also found by Dytham and Weedon (4) who studied the cleavage of 12-oxo- and 12-hydroxystearic acids by KOH at 300C in an open tube. Steadman and Peterson claimed that their product was a mixture of dodecanedioic and undecanedioic acids; however, no trace of the C_{11} dibasic acid was found in the present work and it would not be expected from the explanation of the reaction given by Dytham and Weedon (4). These authors suggested that the first stage was dehydrogenation to the oxo- acid followed by attack on the carbonyl group by hydroxyl ion to give the carbonium ion below. Fission of each of the carbon-carbon bonds



of the oxygenated carbon atom to the same extent gives equimolar amounts of dodecanedioic acid and hexane, and undecanoic and heptanoic acids.

If this mechanism held for the cleavage of 17-hydroxystearic acid equimolar amounts of heptadecanedioic (II) and palmitic (IV) acids would be expected as shown in Fig. 1. However, when 17-hydroxystearic acid was fused with KOH 74% of the product was C_{17} dibasic acid and only about 2% was palmitic acid. The rest was shorter chain dibasic acids and unidentified by-products. It is probable that, in the case of acids with an oxo- group at the penultimate carbon atom, the carbon to methyl group bond is cleaved much more readily than the carbon to methylene group bond. The actual yield of dibasic acids from this type of hydroxy acid is much better than that from 12-hydroxystearic acid.

Crude, distilled, and hydrogenated hydroxy esters obtained from fermentations of "distillate wax" and oleic acid were fused with KOH. As shown in Fig. 1, 17-hydroxyoleic acid (V) loses 3 carbon atoms giving mainly pentadecanedioic acid (VI). Myristic acid (VII), the theoretically possible monobasic product, formed about 5% of the product and the dibasic acids about 80%; the remaining 15% appeared to be a complex mixture of branched chain monobasic acids (4). Hexadecanedioic acid, which was also isolated from the fusion of hydroxy esters from fermented oleic acid, is derived from 16-hydroxypalmitic acid by dehydrogenation and from 18-hydroxyoleic acid by dehydrogenation and loss of two carbon atoms (see Table I for composition of starting material). The composition of the dicarboxylic acid portion of the products and the yields obtained are given in Table II.

The results show that for esters A, which are about 75% saturated, hydrogenation has not had much effect on the quality of the product or its composition; in fact, the loss on distilling crude hydroxy esters makes the percent yield lower for hydrogenated material than for crude material. However, for esters (B), which are 88% unsaturated, before hydrogenation the product contained mainly C_{15} and C_{16} dibasic acids, but after hydrogenation it contained mainly C_{17} and C_{18} dibasic acids; the yield was about 10% higher using hydrogenated esters because greater losses occurred in fission of unsaturated esters.

Comparison of Methods and Starting Material

The nitric acid method is simpler and more readily modified for larger scale preparations, while alkali fusion is accompanied by considerable foaming and requires the use of a reaction vessel with a volume at least ten times greater than that of the reactants. However, for nitric acid oxidation the starting materials have to be hydrogenated since even when saturated compounds are fermented, the hydroxy acids produced always contain about 10–20% of monoenoic substances. If a convenient method of carrying out alkali fusion could be developed, this method would probably be preferred, as it would be easier to use crude hydroxy esters directly. The yield of dibasic acids obtained by the two methods are not very different though the compositions of the products differ since nitric acid oxidation results principally in the loss of two carbon atoms and alkali fusion in the loss of one or three carbons.

Fermentation of hydrocarbons results in conversion of 60–80% of added substrate to hydroxy acids, but in fermentation of fatty acids 75–90% of the substrate is converted (7,9). Aside from this factor and the question of availability and cost of substrate, the choice of fermentation substrate mainly affects the composition of the dibasic acid products. Since odd-numbered hydrocarbons of chain length greater than 19 carbons are converted to 16-hydroxyheptadecanoic acid, and even-numbered hydrocarbons of chain length greater than 18 are converted to 17-hydroxystearic acid (9), any commercial hydrocarbon cut will give hydroxy esters similar to esters (A), in Table I, on fermentation. Therefore, the composition of the dicarboxylic acids, derived from esters (A) and given in Table II cannot be varied much, unless pure C_{16}, C_{17} or C_{18} hydrocarbons are fermented.

However, hydroxy esters produced by fermentation of natural fats are more variable (7,9), and a greater variety of dibasic acids can be produced from them. Thus, as found with hydroxy esters (B), the degree of hydrogenation has a considerable effect. Also, substrates which give rise to much larger amounts of ω-hydroxy acids can be used. Fermentation of fatty acids rich in palmitic acid gives up to 60% of 16-hydroxypalmitic acid (9), which gives good yields of hexadecanedioic acid by both nitric acid oxidation and alkali fusion. When sunflower oil which is high in linoleic acid is fermented, 58% of ω-hydroxylinoleic and -oleic acids are obtained. After hydrogenation, this material could be converted to dibasic acids rich in octadecanedioic acid.

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